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Metabolism of Biochanin A and Formononetin by Human Liver Microsomes in Vitro

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Biochanin A and formononetin are abundant in legumes. These proestrogenic isoflavones can be converted by 4′-*O*-demethylation to the more potent phytoestrogens genistein and daidzein. Incubation of biochanin A or formononetin with human liver microsomes resulted in 4′-*O*-demethylation and the production of additional metabolites. Three new hydroxylated formononetin derivatives, 6,7-dihydroxy-4′-methoxyisoflavone, 7,8-dihydroxy-4′-methoxyisoflavone, and 7,3′-dihydroxy-4′-methoxyisoflavone, were isolated and characterized. We surveyed the *O*-demethylase competence of cytochrome P450 isoforms found in human liver. Human cytochrome P450 isoforms 1A2, 2E1, 2C9*1, 2C19, and 2D6*1 catalyzed biochanin A consumption and genistein production. Human cytochrome P450 isoforms 1A2, 2C9*1, 2A6, 2D6*1, and 2C19 catalyzed formononetin consumption and daidzein production. These isoforms also generated other hydroxylated metabolites. Although *O*-demethylation of isoflavones has been attributed to metabolism by gut microflora, our study demonstrates that human hepatic microsomal enzymes can perform the same transformation and may play a key role in the conversion of 4′-*O*-methylated isoflavones to more potent phytoestrogens.

KEYWORDS: Isoflavone metabolism; cytochrome P450; biochanin A; formononetin

INTRODUCTION

Both protective and adverse health effects have been associated with consumption of foods and food supplements containing isoflavone derivatives. Dietary consumption of isoflavones has been linked to lower risks for breast cancer (*1*) and prostate cancer (*2*), as well as protection from osteoporosis and postmenopausal "hot flashes" (*3*). Isoflavones have also been linked to reproductive dysfunction in rats (*4*, *5*) and male mice (*6*). In addition, they have been found to exert diverse biological effects in different cell types through interacting with estrogen receptors, acting as antioxidant agents, and inhibiting important mammalian enzymes, such as the tyrosine kinase activity of the epidermal growth factor receptor (*7*).

Isoflavones are abundant in legumes such as soy, mung bean sprouts, kidney beans, navy beans, red clover, and Japanese arrowroot (kudzu) (*8*, *9*). Important estrogenic isoflavones available in foods include genistein and daidzein (**Figure 1**). Biochanin A and formononetin are the 4′-*O*-methyl derivatives of genistein and daidzein, respectively, and are the predominant isoflavones found in alfalfa (*9*), red clover (*8*), and chick peas (*8*).

Plant isoflavones are typically detected in food products as substituted glucoside conjugates. The bioavailability and biological activity of isoflavones found in foods typically require hydrolysis of glycosidic bonds to liberate the free aglycons. For instance, deglycosylation to the aglycons genistein and daidzein has been demonstrated in the gut of sheep and cattle fed genistin and daidzin (*10*, *11*). In another study, bacteria isolated from human intestinal microflora have been shown to possess the same capability (*12*). Additional metabolism of biochanin A and formononetin usually involves *O*-demethylation (*11*, *12*), which leads to an increase in the extent of binding to estrogen receptor molecules, presumably through the 4′-hydroxyl group (*13*). However, because the efficiency of anaerobic demethylation by acetogenic bacteria is relatively low, the possibility that human hepatic enzymes may be capable of demethylating the proestrogenic isoflavones biochanin A and formononetin to yield the more estrogenic derivatives genistein and daidzein should be considered. In this study, we demonstrate that human hepatic microsomal enzymes, including several cytochrome P450 isoforms, are quite active in performing these metabolic transformations. In addition, three new formononetin metabolites have been isolated, and they are characterized by ^{1}H NMR and mass spectrometry.

MATERIALS AND METHODS

Chemicals and Biochemicals. Genistein, daidzein, formononetin, and biochanin A were obtained from Indofine Chemical Company

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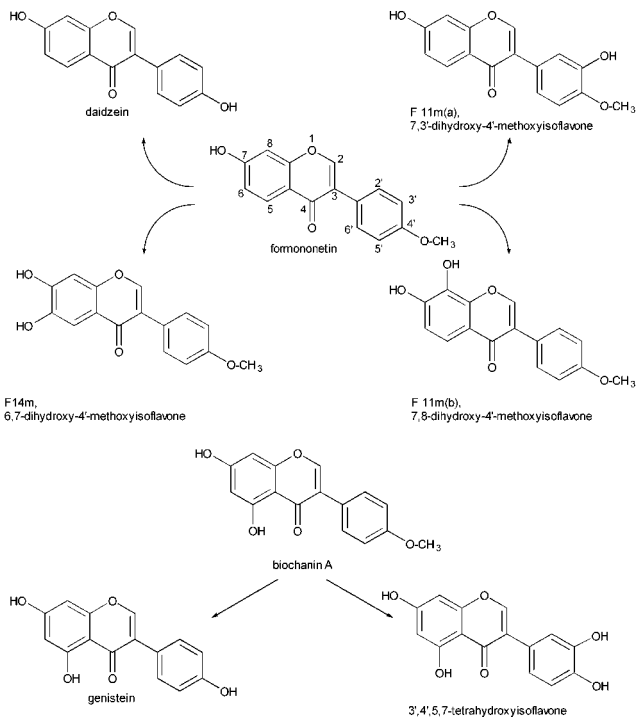


Figure 1. Structures of the characterized formononetin and biochanin A metabolites produced in incubations with human liver microsomes.

(Somerville, NJ). Human liver microsomes (blended from 21 donors), microsomes engineered to express human cytochrome P450 isoforms, and the NADPH regenerating system were obtained from Gentest Corporation (Woburn, MA). Isoflavone stock solutions were prepared in DMSO and diluted to 0.1 mM with absolute ethanol (0.2% DMSO, final). Concentrations of isoflavone stock solutions were determined by UV spectrophotometry using molar absorptivity coefficients reported by Franke and Custer (*14*).

Isoflavone Analysis Using HPLC/EC and HPLC−MS. Analysis was carried out using two model 580 solvent pumps equipped with pulse dampeners and high-pressure mixing; a model 540 autosampler with sample refrigeration set at 4 °C; a temperature-controlled column oven adjusted to 28 °C; a serial array of seven coulometric electrodes (ESA Inc., Chelmsford, MA), and a model 10Avp UV detector (Shimadzu, Columbia, MD). Coularray software V 1.04 and a Pentium-based PC were used to acquire data and control the system. The column was a 50 × 4 mm 3-μm YMC AM with a similar 20 × 4 mm guard column (Waters Corp., Milford, MA). The binary mobile phase consisted of (A) methanol/acetonitrile/water/1 M ammonium acetate, pH 3.8; 1:3:15:1 (v/v/v/v), and (B) methanol/acetonitrile/water/1 M ammonium acetate, pH 3.8; 4:10:5:1 (v/v/v/v). Samples were eluted using a 25-min linear gradient from 0 to 75% B at 1.0 mL/min, followed by a 5-min hold at initial conditions. The coulometric electrodes were set at 0, 140, 280, 420, 560, 700, and 840 mV (vs α-hydrogen/palladium).

Levels of biochanin A, daidzein, genistein, and formononetin in incubation mixtures were determined from standard curves prepared from injections ranging from 5 to 100 pmol of each isoflavone. The response for each of the isoflavones in the standard mixtures was linear regardless of whether quantitation was based on dominant peak area, height, sum of the cluster areas, or UV absorbance at 260 nm ($r >$ 0.999 with the curves forced through zero). Levels of unidentified metabolites were estimated on the basis of Faraday's Law, assuming two-electron reactions and 100% electrolytic efficiency for the flow-through coulometric sensors (*15*). The nature of the products formed from biochanin A and formononetin was investigated using on-line high-pressure liquid chromatography (LC) with electrospray tandem mass spectrometry (LC−ES/MS/MS). LC conditions were comparable to those described above for LC/EC, except that the ammonium acetate buffer was replaced with 0.1% formic acid (final concentration), and a portion of the column effluent (75%) was split to a UV detector (260 nm). A Quattro Ultima triple stage quadrupole mass spectrometer (Micromass, Manchester, U.K.) equipped with an electrospray source was used with an ion source temperature of 150 °C. For MS/MS measurements a collision cell gas pressure (Ar) of $(2-4) \times 10^{-3}$ mbar was used to acquire positive product ions (m/z 300−600), and for MS measurements positive ions were acquired in full scan (m/z 100−400 in 1 s cycle time).

Microsomal Incubations. Reaction mixtures (0.5 mL each) containing 0−1.0 μM isoflavones, 1.0 mg/mL human liver microsomal protein, 50 mM potassium phosphate, 1 mM EDTA, 1.33 mM $NADP^+$, 3.3 mM glucose-6-phosphate, and 3.3 mM magnesium chloride were equilibrated for 3 min at 37 °C and were initiated by adding 0.4 U/mL glucose-6-phosphate dehydrogenase. Incubations were conducted at 37 °C with gentle mixing for the times given in appropriate table and figure legends. The reactions were quenched by extracting two times with 1.0-mL portions of ethyl acetate. The organic extracts were combined, dried in vacuo, and stored at −80 °C. The samples were reconstituted in 75−100 μL of methanol assisted by ultrasonic agitation, and duplicate 20−25 μL aliquots were injected into the HPLC detection system. Recovery of analytes from reaction mixtures was 86 ± 3.6% ($n = 5$), as determined in representative reactions spiked with 20 μL of 5.0 μM estradiol (E2). Similar recoveries were observed from control reactions that did not contain an enzyme source or were extracted immediately after adding the substrate.

Microsomes engineered to contain recombinant human cytochrome P450 isoforms plus human cytochrome P450 reductase (Gentest Corp.) were used to identify human enzymes capable of formononetin and biochanin A metabolism. Each 0.5-mL reaction contained 1.0 μM formononetin or biochanin A, 1.0 mg/mL microsomal protein, and the buffer and NADPH regeneration system described above, with the exception of incubations conducted with CYP2A6 and CYP2C9*1, for which the buffer was 100 mM Tris, pH 7.5, as recommended by the supplier. The human CYP450 contents of the microsomal preparations reported by the supplier were: 170 pmol CYP1A2/mg protein, 202 pmol CYP2A6/mg protein, 125 pmol CYP2B6/mg protein, 45 pmol CYP2C9*1/mg protein, 58 pmol CYP2C19/mg protein, 164 pmol CYP2D6*1/mg protein, 200 pmol CYP2E1/mg protein, and 70 pmol CYP3A4/mg protein. The reactions were incubated for 3 h at 37 °C, and then extracted twice with ethyl acetate, dried, reconstituted in methanol, and analyzed by HPLC as described above.

Rat Liver Microsome Reaction. To obtain quantities of hydroxylated formononetin metabolites sufficient for ^{1}H NMR analysis, a 100-mL reaction was prepared containing 10 μM formononetin, 10 mg/mL β-naphthoflavone-induced rat liver microsomes (In Vitro Technologies, Baltimore, MD), and the same NADPH generating system and buffer described above, and incubated for 3 h at 37 °C. Cold acetonitrile, 100 mL, was added to the reaction mixture, which was incubated at 0 °C for 25 min to precipitate proteins, then 70 mL of hexane and 10 mL of dichloromethane were added to the clear supernatant. This solution was mixed by shaking in a 250-mL separatory funnel and allowed to settle into three phases. The middle organic phase was evaporated to dryness under vacuum and the yellow/white residue was dissolved in 7 mL of a solvent of acetonitrile/DMSO/water (4:1:2). This material was diluted to 100 mL by addition of 93 mL of 25 mM ammonium acetate, pH 3.8, containing 5% MeOH (v/v, conditioning buffer). Isoflavones were isolated by solid-phase extraction using a Sep-Pak cartridge (C18, 6 cm^3, 1 g; Waters) preconditioned with 10 mL of MeOH followed by 10 mL of conditioning buffer. The 100-mL fraction was applied to the SPE column, and the column was washed with 10 mL of conditioning buffer and then eluted with two applications of 2 mL of MeOH. The combined eluant was evaporated to dryness, and the residue was dissolved in 0.16 mL of DMSO. Fractions containing the F 11m and F 14m metabolites were obtained by preparative HPLC (50 × 4 mm 3 μm YMC AM) with detection by UV at 260 nm. Pooled fractions were evaporated to dryness under vacuum and dissolved in 100 μL of MeOH. Metabolite F11m(a) was baseline resolved from F11m(b) using isocratic elution with 20% B, and the center cut fractions were pooled. Volatile buffer and solvent components were removed under vacuum. The F 11m(a), F 11m(b), and F 14m metabolites (vide infra) produced from rat liver microsomes had LC retention times and oxidation profiles

identical to those of the F 11m(a), F 11m(b), and F 14m metabolites produced from human liver microsomes or from recombinant human CYP1A2.

^{1}H NMR Analyses. Analyses of the formononetin metabolites isolated from rat liver microsomal incubations were performed both in acetone-d_6 and methanol-d_4 solutions, on a Varian Unity 300 spectrometer operated at 300 MHz, using a 5-mm indirect detection probe. Chemical shifts are reported in ppm downfield from tetramethylsilane and coupling constants are reported in Hz. Proton assignments were based on comparison with the spectrum of formononetin, combined with homonuclear decoupling experiments.

Regression Analysis. Michaelis−Menten enzyme kinetic constants were determined using Sigma Plot (Jandel Scientific, San Rafael, CA) for the metabolism of isoflavone substrates catalyzed by human liver microsomal enzymes.

RESULTS

Isoflavone Metabolites Formed by *O*-Demethylation and Hydroxylation. Organic extracts from incubations of formononetin or biochanin A in the presence of human liver microsomes were analyzed chromatographically using coulometric detection. The structures of the isoflavone substrates and characterized products are depicted in **Figure 1**. The primary products from formononetin incubations were daidzein, which was identified by comparison of its mass spectrum, LC retention time, and electrochemical oxidation profile to those of authentic daidzein, and two additional metabolites: one that eluted at 13.8 min and was designated F 14m, and another that eluted at 10.8 min and was designated F 11m. Further analysis indicated that the F 11m peak was actually composed of two closely eluting metabolites; of these, the minor one eluted slightly earlier and was labeled F 11m(a), and the major one was labeled F 11m(b). The HPLC/EC analysis of a typical 3-h incubation of 1.0 μM formononetin with human liver microsomes is presented in **Figure 2A**. The amounts of formononetin (28% remaining) and its metabolites (daidzein 34.6%; F 14m, 23%; and total F 11m, 11.7%) were estimated relative to the amount of initial formononetin substrate.

The three unknown formononetin metabolites, F 11m(a), F 11m(b), and F14m, were analyzed by LC−ES/MS/MS, which revealed in each instance a protonated molecule (*m*/*z* 285), a mass consistent with that of hydroxylated formononetin derivatives that maintained the 4′-*O*-methyl group. Product ion spectra for the metabolites contained a similar, but not identical, pattern of fragment ions consistent with an assignment of isomeric hydroxylated formononetins (*m*/*z* 269, 253, 242, 229, 213, 211, 196, 183, 170, 168, 152, 133, 123). Further characterization of the three metabolites by ^{1}H NMR spectroscopy (**Table 1**) confirmed this assignment. Thus, a three-proton methyl singlet, with a chemical shift (ca. 3.8 ppm) virtually identical to that of 4′-*O*-methyl group in the parent formononetin, was clearly observed for each of the metabolites. The basic isoflavone structure was also conserved, as indicated by the presence of a downfield singlet corresponding to the H2 proton (ca. 8.1−8.2 ppm), combined with the absence of any protons ascribable to saturated carbons. Therefore, none of the three metabolites corresponded to known reduced formononetin metabolites, such as equol or *O*-desmethylangolensin (*14*, *15*). Analysis of the signals corresponding to the aromatic protons in F 11m(a) indicated that the chromone substitution profile was identical to that of formononetin, with the H5, H6, and H8 protons displaying the patterns expected for ortho (H5−H6) and meta (H6−H8) coupled protons (**Table 1**). By contrast, the *O*-methylated ring contained only three almost superimposed protons, at approximately 7.0 ppm. This is fully consistent with

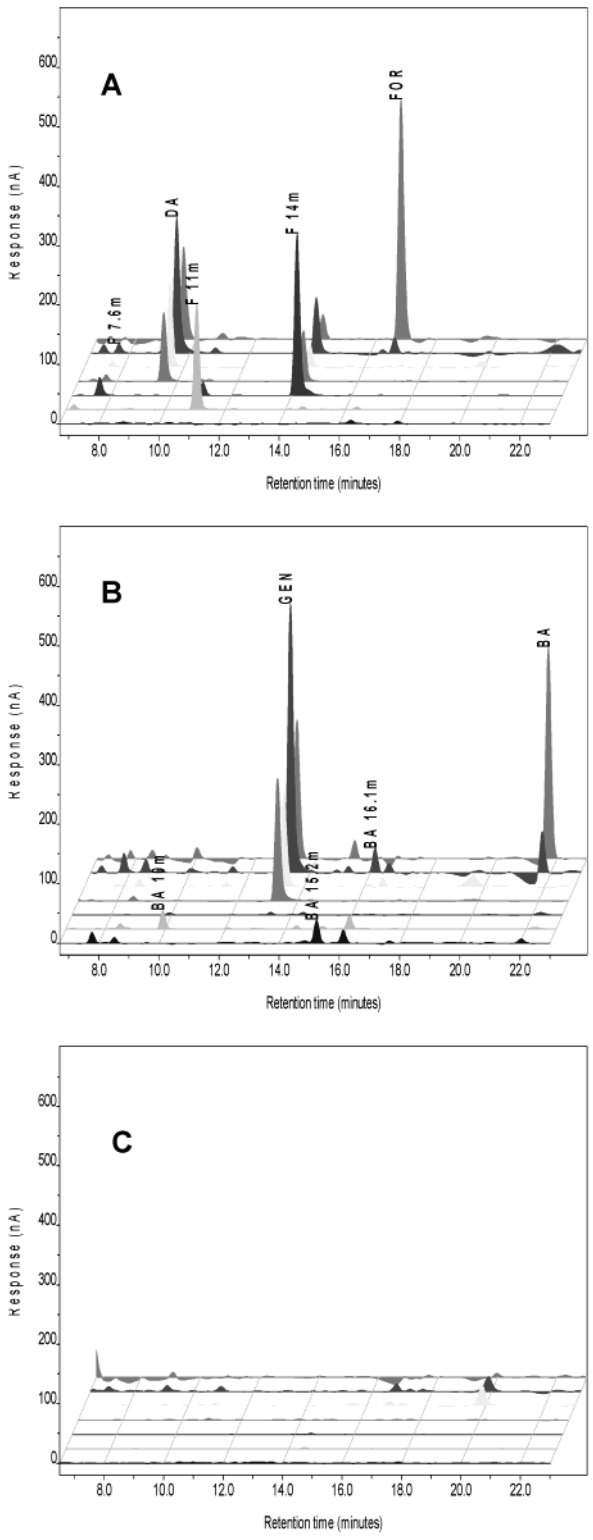
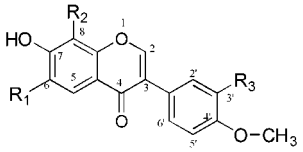


Figure 2. (A) Multichannel HPLC/EC chromatograms of the products of 3-h incubations of 4′-*O*-methylated isoflavones with human liver microsomes. Formononetin (FOR) produced daidzein (DA), two major hydroxylated metabolites (F 11m and F 14m), and a minor unidentified metabolite, F 7.6m. (B) Biochanin A (BA) produced genistein (GEN) and two minor unidentified metabolites (BA 15.2m and BA 16.1m). (C) Chromatogram for a 3-h incubation control that included human liver microsomes and the NADPH generating system but no isoflavone substrate. Chromatograms are shown at 700 nA full scale; the potentials of the detector cells in the coulometric array were 0, 140, 280, 420, 560, 700, and 840 mV.

Table 1. ^{1}H NMR Data for Formononetin and Hydroxylated Formononetin Metabolites[a]



δ (ppm)	formonetin (R_1=R_2=R_3=H)	F 11m(a) (R_1=R_2=H, R_3=OH)	F 11m(b) (R_1=R_3=H, R_2=OH)	F 14m (R_2=R_3=H, R_1=OH)
CH_3	3.82 (3H, s)	3.87 (3H, s)	3.82 (3H, s)	3.82 (3H, s)
H2	8.16 (1H, s)	8.09 (1H, s)	8.20 (1H, s)	8.10 (1H, s)
H5	8.05 (1H, d, *J* 8.7)	8.00 (1H, d, *J* 8.4)	7.57 (1H, d, *J* 9.0)	7.43 (1H, s)
H6	6.93 (1H, dd, *J* 8.7, *J′* 1.8)	6.87 (1H, dd, *J* 8.7, *J′* 1.8)	6.94 (1H, d, *J* 9.0)	
H8	6.85 (1H, d, *J* 1.8)	6.76 (1H, d, *J* 1.8)		6.82 (1H, s)
H2′,6′	7.46 (2H, d, *J* 8.7)		7.47 (2H, d, *J* 8.7)	7.45 (2H, d, *J* 8.7)
H3′,5′	6.98 (2H, d, *J* 8.7)		6.98 (2H, d, *J* 8.7)	6.98 (2H, d, *J* 8.7)
H2′		7.03 (1H, s)		
H5′		6.97 (1H, bs)		
H6′		6.97 (1H, bs)		

[a] The reported data were obtained in methanol-d_4. Abbreviations: bs, broad singlet; d, doublet; dd, doublet of doublets, s, singlet, *J*, coupling constant (Hz).

hydroxylation at C3′, as hydroxylation at C2′ would be expected to have no significant effect on H6′, which should display a chemical shift of approximately 7.5 ppm, as observed in formononetin (**Table 1**). Taken together, these data indicate that metabolite F 11m(a) was 7,3′-dihydroxy-4′-methoxyisoflavone. Contrasting with F 11m(a), both F 11m(b) and F 14 m had an intact 3-(4′-*O*-methoxy)phenyl ring, as indicated by the presence of two mutually coupled two-proton doublets at approximately 7.0 and 7.5 ppm, essentially identical to those of formononetin. On the basis of the presence of two mutually ortho-coupled one-proton doublets (**Table 1**), the F 11m(b) metabolite was readily identified as 7,8-dihydroxy-4′-methoxyisoflavone. Similarly, the presence of two one-proton singlets at ca. 7.4 (H5) and 6.8 (H8) ppm, with no evidence of meta-coupling (**Table 1**), led to the identification of F 14m as 6,7-dihydroxy-4′-methoxyisoflavone. Should hydroxylation have occurred at C5, both signals would be expected to be located more upfield.

In a manner similar to that described for formononetin, 1.0 μM biochanin A was incubated with human liver microsomes, and the metabolites were extracted and analyzed by HPLC/EC as depicted in **Figure 2B** for a representative 3.0-h reaction. The major metabolite of biochanin A was genistein, which was identified by comparison of its mass spectrum, LC retention time, and electrochemical oxidation profile to those of authentic genistein. Incubation of biochanin A with human liver microsomes also produced metabolites that eluted at 10, 15.2, and 16.1 min and were designated BA 10m, BA 15.2m, and BA 16.1m. The BA 10m metabolite had a protonated molecule with *m*/*z* 287 and the fragmentation was consistent with identification of this metabolite as 3′,4′,5,7-tetrahydroxyisoflavone as previously reported (*16*, *17*). Both the BA 15.2m and BA 16.1m biochanin A metabolites had a protonated molecule with *m*/*z* 301, consistent with their being hydroxylated biochanin A, but regioisomers could not be determined from the fragmentation data alone. On the basis of the sums of the coulometric cluster areas, the amounts of biochanin A and its metabolites detected after the 3.0-h reaction were the following: biochanin A, 31.3%; genistein, 60.4%; BA 15.2m, 2.9%; and BA 16.1m, 3.2%; relative to the initial amount of biochanin A substrate.

The time-course curves for substrate depletion and product formation are depicted in **Figure 3** for formononetin (**panel A**) and biochanin A (**panel B**) incubations with human liver microsomes. Note that the daidzein and the major hydroxylated

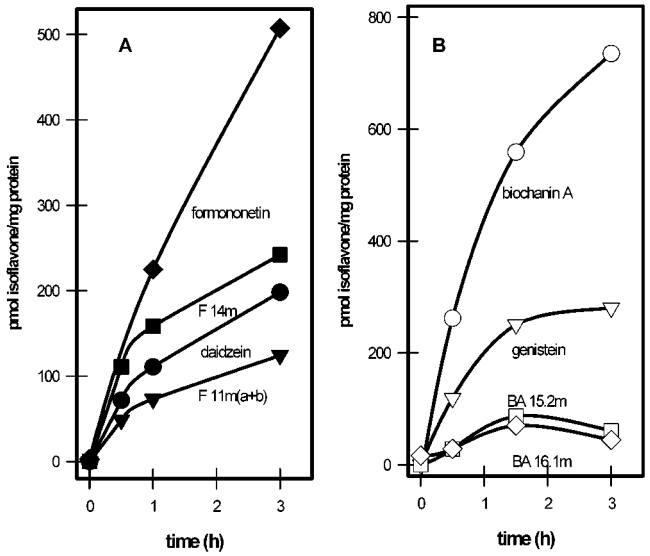


Figure 3. Time-course curves for the metabolization of formononetin and biochanin A catalyzed by human liver microsomes. (A) Human liver microsomes were incubated with 500 pmol formononetin; and the amounts of daidzein (●), and formononetin derivatives F 11m (combined F 11m(a) and F11m(b), ▼) and F14 m (■), formed per mg of microsomal protein are plotted. The amounts of formononetin (♦) consumed per mg of protein are also plotted. (B) Human liver microsomes were incubated with 500 pmol biochanin A, and the amounts of genistein (▽), and biochanin A derivatives BA 15.2m (□) and BA 16.1m (◇), formed per mg of microsomal protein are plotted. The amounts of biochanin A (○) consumed per mg of protein are also plotted.

formononetin metabolites formed accounted for approximately 97% of the observed formononetin depletion. Two additional minor uncharacterized formononetin metabolites, labeled F 6.9m and F 7.6m (**Figure 2** and **Table 2**), are likely to account for the remaining formononetin depletion. Although 4′-*O*-demethylation of biochanin A to yield genistein represented the predominant reaction, the amount of biochanin A consumed was greater than the amount of genistein formed. The accumulation of BA 15.2 and BA 16.1 appeared to be transient, as these metabolites were in greater abundance in 1.5-h reactions than in 3-h reactions (**Figure 3B**). We suspect that additional

Table 2. Apparent Michaelis–Menten Kinetic Constants for Human Liver Microsomal Metabolism of Formononetin and Biochanin A

reaction	K_m (μM)	V_{max} (pmol/min mg protein)
formononetin depletion	15.4	138
daidzein synthesis	3.06	3.19
F 11m synthesis (combined F 11m(a) and F 11m(b))	2.46	3.39
F 14m synthesis	1.48	5.64
biochanin A depletion	9.34	108
genistein synthesis	14.0	25.0

biochanin A metabolites, not detected by our chromatographic technique, were formed.

The rate of product formation and substrate consumption over time appeared in both instances to be linear at 1 h, and subsequent experiments to determine Michaelis−Menten constants were performed using 1-h incubations. The apparent K_m and V_{max} values for the consumption of the two *O*-methylated isoflavone substrates, the formation of the demethylated products, and the formation of the new hydroxylated formononetin metabolites are given in **Table 2**. Because multiple enzymes present in human liver microsomes were later found competent to produce the formononetin and biochanin A metabolites, the observed variations in enzymatic parameters for each metabolite were consistent with the complexity of the reaction. In subsequent experiments with single cytochrome P450 isoforms, we observed that both the mix and relative preponderance of the metabolites varied as a function of hepatic cytochrome P450 isoform. Thus, the apparent enzyme kinetic parameters given in **Table 2** represent the composite nature of the blended human liver microsome preparations used in these experiments and reflect the different reaction-pathways enzymes contributing to the observed products.

Metabolism of Formononetin and Biochanin A by Various Isoforms of Human Cytochrome P450 Found in Liver. To determine which isoforms of CYP450 were responsible for the *O*-demethylation of biochanin A and formononetin, we surveyed the principal individual isoforms reported to be expressed in the human liver microsome pool. Extracts of the incubations were analyzed by HPLC/EC and chromatograms representing the isoforms that exhibited *O*-demethylation activity are shown in **Figures 4** and **5**, together with those of selected controls. For the series of experiments represented in **Figures 4** and **5**, 3-h incubations contained 500 pmol of biochanin A or formononetin or no substrate, and an NADPH generating system. Except as noted, the incubations also contained specific CYP450 isoforms, each at a concentration concordant with the concentration of that isoform in the human liver microsome pool, as indicated in the legends to **Figures 4** and **5**. The exception was the 2D6 isoform, which was used at 500 pmol per 0.5-mL reaction, a concentration approximately 53-fold higher than that found in the human liver microsome pool. Protein concentrations were adjusted to 1.0 mg/mL using microsomes devoid of detectable CYP450 activity (Gentest). In a subsequent series of incubations, the amount of each cytochrome P450 isoform was 50 pmol per reaction, incubations were conducted for 1 h, and the substrate was either 500 pmol formononetin (**Figure 6A**) or 500 pmol biochanin A (**Figure 6B**). The number and identity of metabolites produced using a particular isoform and substrate were reproducible, regardless of isoform concentration and incubation time.

The potencies of P450 isoforms to produce daidzein by *O*-demethylation of formononetin were as follows (expressed as pmol daidzein formed per hour per pmol CYP450): 1A2 (1.488) > 2C9*1 (0.540) > 2A6 (0.101) > 2D6*1 (0.075) > 2C19 (0.061). The formononetin metabolites 7,3′-dihydroxy-4′-methoxyisoflavone (F 11m(a)), 7,8-dihydroxy-4′-methoxyisoflavone (F 11m(b)), and 6,7-dihydroxy-4′-methoxyisoflavone (F 14m) were produced to the greatest extent in the CYP1A2 incubations, in which they were present at levels (estimated from dominant channel area) comparable to the level of daidzein. 7,3′-Dihydroxy-4′-methoxyisoflavone and 6,7-dihydroxy-4′-methoxyisoflavone were present as trace metabolites in CYP 2C9*1, 2C19, and 2D6*1 incubations (**Figures 4** and **6A**).

Similarly, the potencies of each CYP450 isoform to produce genistein from biochanin A were as follows (expressed as pmol genistein formed per hour per pmol CYP450): 1A2 (1.072) > 2E1 (0.496) > 2C9*1 (0.298) > 2C19 (0.108) > 2D6*1 (0.0468) > 2A6 (0.027). The minor metabolites BA 15.2m and BA 16.1m were detected in the 1A2 incubations, and BA 16.1m was detected at low levels in CYP 2D6, CYP 2C9, CYP 2D6*1, and CYP 2E1 (**Figure 5A**, **Figure 6B**).

It should be noted that additional minor peaks were also observed in the corresponding 'no substrate' controls and were, therefore, disregarded as isoflavone metabolites. The peak eluting at approximately 19.5 min, shown in **Figure 5F**, is an example of these unknown species. No metabolism of biochanin A or formononetin was detected in incubations containing CYP450 isoform 3A4.

DISCUSSION

The detection of the estrogenic isoflavones genistein and daidzein in the urine, feces, breast milk, and blood of animals and humans following the consumption of isoflavone-containing foods demonstrates their bioavailability through the diet. Lundh et al. (*18*) detected genistein and daidzein along with an estrogenic metabolite of daidzein, equol, and the proestrogenic isoflavone formononetin in the plasma and urine of a dairy cow fed a normal diet containing isoflavones. Dickinson et al. (*11*) measured the rate of formononetin consumption in the ruminal fluid of a fistulated steer fed alfalfa hay as an isoflavone source. The concentration of formononetin declined from 14.80 μg/mL to 1.16 μg/mL at 12 h and further to 0.76 μg/mL by 24 h in ruminal fluid while daidzein and equol levels increased, demonstrating that gastric microorganisms of cattle can effect isoflavone *O*-demethylation and also have isoflavone reductase activity. Breinholt et al. (*19*) administered biochanin A, genistein, daidzein, and equol dissolved in DMSO by gavage to 6-week-old Arochlor-1254-treated female mice and measured the levels of isoflavone metabolites in the urine and feces over time. Their study showed that 90.1% of the equol dose appeared in the urine, 78.6% as the unmetabolized aglycon. In contrast, only a total of 32% of the added biochanin A dose was detected as either biochanin A, genistein, or hydroxylated metabolites of these two isoflavones in the urine and feces combined. Biochanin A was apparently metabolized to other undetected products or deposited in other tissues. Similar results were observed with genistein and daidzein, for which the combined urinary and fecal recoveries were 29% and 60%, respectively. Setchell et al. (*20*) measured the concentrations of genistein, daidzein, formononetin, and biochanin A in the plasma of human volunteers consuming commercially available clover-based dietary supplements as an isoflavone source. Following the consumption of a food supplement product providing 14 mg of formononetin and 27 mg of biochanin A, plasma levels of these isoflavones peaked at 10 nM and 30 nM, respectively, shortly after administration. Extensive metabolic demethylation was apparent because the levels of daidzein and genistein peaked at

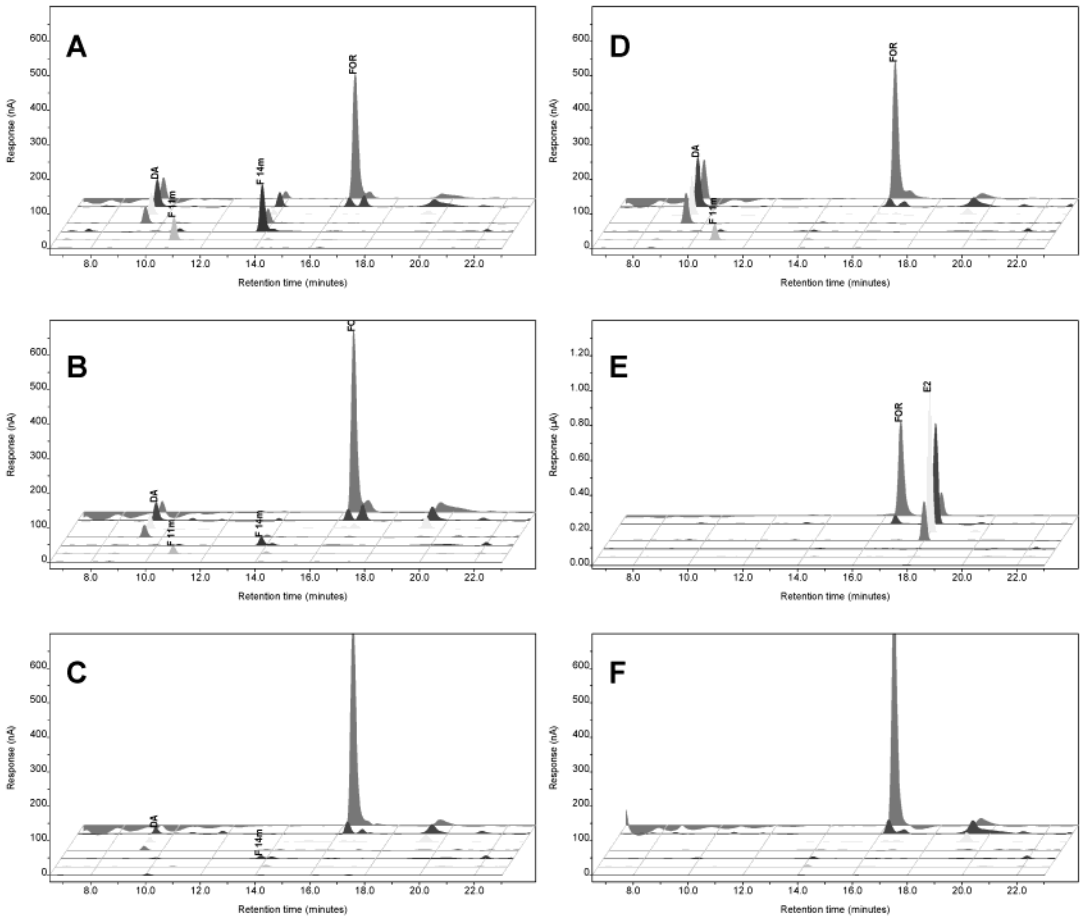


Figure 4. Chromatograms of extracts of 3-h, 0.5-mL incubations containing 500 pmol formononetin and microsomes containing specific recombinant human P450 isoforms (amount) and human cytochrome P450 reductase: (A) 1A2 (85 pmol); (B) 2C9 (45 pmol); (C) 2C19 (29 pmol); (D) 2D6*1 (500 pmol); and (E) 2E1 (90 pmol). (F) represents an incubation of formononetin with microsomes from the parental AHH-1 cell line (OR) overexpressing P450 reductase, but not transfected with the c-DNA for any human P450 isoform. The mixture represented in panel E contained estradiol (E2), which was added as an internal standard after the incubations were quenched.

60 nM and 120 nM, respectively, 4 h after ingestion of the 4′-methoxyisoflavone precursors.

The detection of formononetin or biochanin A metabolites in blood, urine, or feces does not indicate the site of metabolic action. Previously, conversion of biochanin A to genistein, and formononetin to daidzein, has been attributed to metabolism by gut microflora. The data presented here indicate that the liver should be considered as a potentially major site for *O*-demethylation of phytoestrogens. Previously, microorganisms found in the gut, such as *Eubacterium limosum* and *Escherichia coli* HGH6 (*12*), have been shown to be capable of certain metabolic transformations of *O*-methylisoflavones, but required incubation times of several days (*21*). The data presented here demonstrate for the first time that human liver microsomal enzymes are capable of catalyzing hydroxylation and 4′-*O*-demethylation reactions of formononetin and biochanin A. Roberts-Kirchhoff et al. (*22*) reported K_m values ranging from 16 to 104 μM for the generation of oxidative metabolites of genistein catalyzed by pyridine-induced rat liver microsomes. We report K_m values ranging from 1.48 to 15.4 μM for the generation of formononetin and biochanin A metabolites by human liver microsomal enzymes. We also show that human hepatic CYP450 isoforms including CYP 1A2, 2A6, 2E1, and 2D6*1, are capable of converting biochanin A to genistein, 3′,4′,5,7-tetrahydroxyisoflavone, two hydroxylated biochanin A metabolites, and several uncharacterized metabolites. Our study further shows that human microsomal enzymes CYP 1A2, 2A6, 2C9*1, 2C19, and 2D6*1 are capable of converting formononetin to daidzein, and, with varying degrees of efficiency, three hydroxylated formononetin derivatives: 6,7-dihydroxy-4′-methoxyisoflavone, 7,8-dihydroxy-4′-methoxyisoflavone, and 7,3′-dihydroxy-4′-methoxyisoflavone. Our observation that human CYP1A2 possesses the greatest isoflavone hydroxylation activity is consistent with the results of Roberts-Kirchhoff et al. (*22*) who found that human CYP1A1 and 1A2 exhibited the highest genistein 3′-hydroxylation activity in their study. The observation that CYP 2E1 has the ability to perform *O*-demethylation of biochanin A to genistein but not of formononetin to daidzein was reproducible, but the reasons for this substrate selectivity remain unclear.

The finding that biochanin A and formononetin, which are found in foods such as garbanzo beans (*8*) and in clover-based health food supplements, can be metabolized by human liver microsomal enzymes to the more estrogenic derivatives genistein and daidzein suggests that this pathway may contribute to the cancer chemoprevention associated with dietary consumption of isoflavones. Whereas intestinal demethylation of methylated isoflavones is an important pathway in ruminants, it is probably not as relevant in human or rodent systems except under special circumstances. Future experiments using germ-free animals consuming biochanin A and/or formononetin would help determine the relative importance of liver and gut microflora in the 4′-*O*-demethylation of these isoflavones. These results may be of particular significance for individuals with atypical

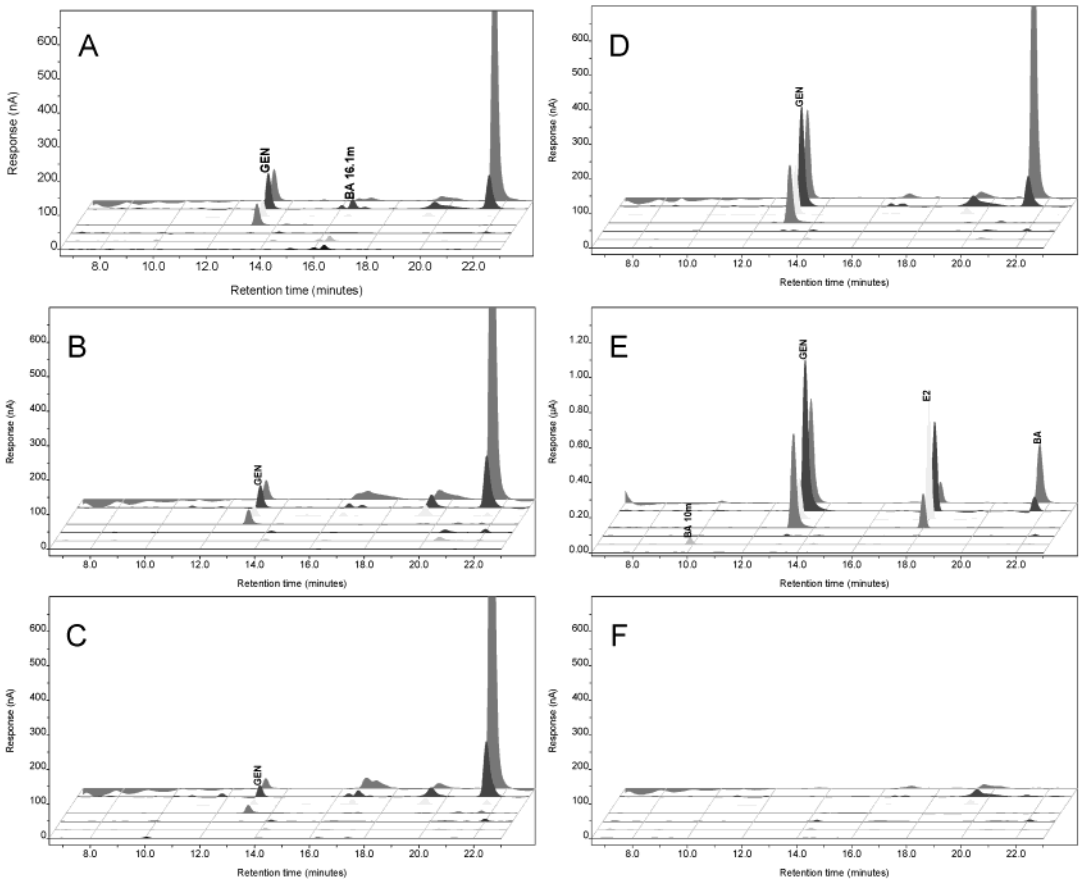


Figure 5. Chromatograms of extracts of 3-h, 0.5-mL incubations containing 500 pmol biochanin A and microsomes containing specific recombinant human P450 isoforms (amount) and human cytochrome P450 reductase: (A) 1A2 (85 pmol); (B) 2C9 (45 pmol); (C) 2C19 (29 pmol); (D) 2D6*1 (500 pmol); and (E) 2E1 (90 pmol). (F) represents an incubation of a P450 isoform (1A2) and P450 reductase without isoflavone substrate. The mixture represented in panel E contained estradiol (E2), which was added as an internal standard after the incubations were quenched.

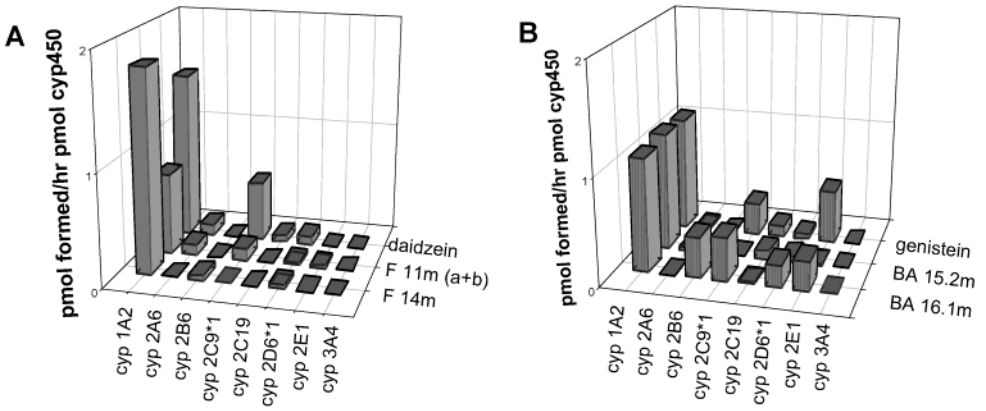


Figure 6. Formation of formononetin and biochanin A metabolites by cytochrome P450 isoforms expressed in human liver: (A) 500 pmol formononetin was incubated for 1 h with microsomes containing 50 pmol of individual cytochrome P450 isoforms; (B) 500 pmol biochanin A was incubated for 1 h with microsomes containing 50 pmol of individual cytochrome P450 isoforms.

populations of intestinal microflora due to infections or use of antibiotics. Furthermore, they also raise the possibility for altered drug metabolism in individuals consuming large amounts of isoflavones. Finally, the detection of isoflavone metabolites in the urine and feces, and the demonstration that both intestinal microorganisms and hepatic enzymes are competent to produce some of the metabolites, does not exclude the possibility of extrahepatic metabolism of isoflavones, particularly in the kidney and intestines, or in target tissues sensitive to estrogen carcinogenesis. Consistent with the concept of extrahepatic metabolism of phytoestrogens in estrogen sensitive target tissues, metabolism of genistein and biochanin A has been demonstrated in human breast cancer cell lines (*23*). Although beyond the scope of the current manuscript, work elucidating the extrahepatic metabolism of biochanin A and formononetin by specific human cytochrome P450 isoforms will be the subject of a future study.

LITERATURE CITED

(1) Lamartiniere, C. A.; Murrill, W. B.; Manzolillo, P. A.; Zhang, J. X.; Barnes, S.; Zhang, X.; Wei, H.; Brown, N. M. Genistein alters the ontogeny of mammary gland development and protects

against chemically-induced mammary cancer in rats. *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 358−364.
(2) Adlercreutz, C. H.; Goldin, B. R.; Gorbach, S. L.; Hockerstedt, K. A.; Watanabe, S.; Hamalainen, E. K.; Markkanen, M. H.; Makela, T. H.; Wahala, K. T. Soybean phytoestrogen intake and cancer risk. *J. Nutr.* **1995**, *125* (suppl), 757S−770S.
(3) Cassidy, A.; Bingham, S.; Setchell, K. D. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am. J. Clin. Nutr.* **1994**, *60*, 333−340.
(4) Awoniyi, C. A.; Roberts, D.; Veeramachaneni, D. N.; Hurst, B. S.; Tucker, K. E.; Schlaff, W. D. Reproductive sequelae in female rats after in utero and neonatal exposure to the phytoestrogen genistein. *Fertil. Steril*. **1998**, *70*, 440−447.
(5) Nagao, T.; Yoshimura, S.; Saito, Y.; Nakagomi, M.; Usumi, K.; Ono, H. Reproductive effects in male and female rats of neonatal exposure to genistein. *Reprod. Toxicol.* **2001**, *15*, 399−411.
(6) Delclos, K. B. Evaluation of phytoestrogen safety and toxicity in rodent models that include developmental exposure. In *Phytoestrogens and Health;* Gilani, G. S., Anderson, J., Eds. AOCS Press: Champaign, IL; in press.
(7) Akiyama, T.; Ishida, J.; Nakagawa, S.; Ogawara, H.; Watanabe, S.; Itoh, N.; Shibuya, M.; Fukami, Y. Genistein: a specific inhibitor of tyrosine-specific protein kinases. *J. Biol. Chem.* **1987**, *262*, 5592−5595.
(8) Mazur, W. M.; Duke, J. A.; Wähälä, K.; Rasku, S.; Adlercreutz, H. Isoflavonoids and lignans in legumes: nutritional and health aspects in humans. *J. Nutr. Biochem.* **1998**, *9*, 193−200.
(9) Horn-Ross, P. L.; Barnes, S.; Lee, M.; Coward, L.; Mandel, J. E.; Koo, J.; John, E. M.; Smith, M. Assessing phytoestrogen exposure in epidemiologic studies: Development of a database (United States). *Cancer Causes Control* **2000**, *11*, 289−298.
(10) Nilsson, A.; Hill, J. L.; Davids, H. L. An in vitro study of formononetin and biochanin A metabolism in rumen fluid from sheep. *Biochim. Biophys. Acta* **1967**, *148*, 92−98.
(11) Dickinson, J. M.; Smith, G. R.; Randel, R. D.; Pemberton, I. J. In vitro metabolism of formononetin and biochanin A in bovine rumen fluid. *J. Anim. Sci.* **1988**, *66*, 1969−1973.
(12) Hur, H. G.; Lay, J. O., Jr.; Beger, R. D.; Freeman; J. P.; Rafii, F. Isolation of human intestinal bacteria metabolizing the natural isoflavone glycosides daidzin and genistin. *Arch. Microbiol*. **2000**, *174*, 422−428.
(13) Kuiper, G. G.; Lemmen, J. G.; Carlsson, B.; Corton, J. C.; Safe, S. H.; van der Saag, P. T.; van der Burg, B.; Gustafsson, J. A. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology* **1998**, *139*, 4252−4263.
(14) Franke, A. A.; Custer, L. J. High-performance liquid chromatographic assay of isoflavonoids and coumestrol from human urine. *J. Chromatogr. B Biomed. Appl.* **1994**, *662*, 47−60.
(15) Gamache, P. H.; Acworth, I. N. Analysis of phytoestrogens and polyphenols in plasma, tissue, and urine using HPLC with coulometric array detection**.** *Proc. Soc. Exp. Biol. Med.* **1998**, *217*, 274−280.
(16) Kulling, S. E.; Honig, D. M.; Simat, T. J.; Metzler, M. Oxidative in vitro metabolism of the soy phytoestrogens daidzein and genistein. *J. Agric. Food Chem*. **2000**, *48*, 4963−4972.
(17) Kulling, S. E.; Honig, D. M.; Metzler, M. Oxidative metabolism of the soy isoflavones daidzein and genistein in humans in vitro and in vivo. *J. Agric. Food Chem*. **2001**, *49*, 3024−3033.
(18) Lundh, T. J.; Pettersson, H.; Kiessling, K. H. Liquid chromatographic determination of the estrogens daidzein, formononetin, coumestrol, and equol in bovine blood plasma and urine. *J. Assoc. Off. Anal. Chem*. **1988**, *71*, 938−941.
(19) Breinholt, V.; Hossaini, A.; Svendsen, G. W.; Brouwer, C.; Nielsen, E. Estrogenic activity of flavonoids in mice. The importance of estrogen receptor distribution; metabolism; and bioavailability. *Food Chem. Toxicol*. **2000**, *38*, 555−564.
(20) Setchell, K., D.; Brown, N. M.; Desai, P.; Zimmer-Nechemias, L.; Wolfe, B. E.; Brashear, W. T.; Kirschner, A. S.; Cassidy, A.; Heubi, J. E. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. *J. Nutr*. **2001**, *131* (suppl), 1362S−1375S.
(21) Hur H.; Rafii, F. Biotransformation of the isoflavonoids biochanin A, formononetin, and glycitein by *Eubacterium limosum. FEMS Microbiol. Lett*. **2000**, *192*, 21−25.
(22) Roberts-Kirchhoff, E. S.; Crowley, J. R.; Hollenberg, P. F.; Kim, H. Metabolism of genistein by rat and human cytochrome P450s*. Chem. Res. Toxicol*. **1999**, *12*, 610−616.
(23) Peterson, T. G.; Ji, G. P.; Kirk, M.; Coward, L.; Falany, C. N.; Barnes, S. Metabolism of the isoflavones genistein and biochanin A in human breast cancer cell lines. *Am. J. Clin. Nutr.* **1998**, *68* (Suppl), 1505S−1511S.

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